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54 **Method for detecting metals.**

57 Metals such as Ca^{2+} , Sr^{2+} , Sm^{3+} , Cd^{2+} and lanthanoids such as La^{3+} , Tb^{3+} and Yb^{3+} are detected by measuring the luminescence produced in contact with reproduced aequorin obtained by adding coelenterazine or its analogues to an enzyme of aequorin (apoaequorin).

EP 0 245 093 A1

-1-

1 METHOD FOR DETECTING METALS

 This invention relates to a method for detecting metals and especially to such a method in which a photo-
5 protein is used, namely an enzyme of aequorin (apoaequorin), together with a substrate, as an emitter, namely coelenterazine or its analogues.

 Aequorin is a photoprotein obtainable from luminescent jellyfish which grow in the northern part of
10 the west coast of America. When one molecule of aequorin is specifically bound to two or three molecules of Ca^{2+} , it oxidises the coelenterazine present, which acts as an emitter to produce luminescence. Thus, natural aequorin exists in the package state containing the enzyme
15 (apoaequorin), the substrate (coelenterazine) and molecular oxygen (O_2). When Ca^{2+} is added to natural aequorin, its reaction with the Ca^{2+} -binding sites present causes the coelenterazine to be oxidised, so that oxycoelenterazine, CO_2 and $h\nu$ (emission) are produced. This light emission
20 can be detected by a photomultiplier and its sensitivity is so high that trace concentrations of Ca^{2+} of about 10^{-7}M are measurable.

 The habitat of the jellyfish which produce natural aequorin is limited to the aforesaid coastal
25 region and the season in which they are produced is also

1 limited. The yield of natural aequorin is only about
10 mg per 50,000 jellyfish. It is thus very important to
obtain aequorin, but a certain supply cannot be assured.

5 The inventor of the present invention has cloned
the cDNA for aequorin obtained from these jellyfish by a
recombinant DNA method. This cDNA clone was named plasmid
pAQ440 (Japanese Patent Application 59-176125). Then, a
plasmid containing a promoter in which the pAQ440 gene was
10 inserted was transformed in a bacterium and it was found
that the natural and fused type of aequorin (apoaequorin)
could be efficiently expressed in E.coli (Japanese Patent
Application 60-280259). This method provided a stable
supply of aequorin.

15 The present invention further improves the
method for detecting Ca^{2+} concentrations using natural
aequorin and also provides a method for detecting metals,
such as Ca^{2+} and others, using apoaequorin biosynthesised
in E.coli.

20 According to one aspect of the present invention,
a method for detecting metals is provided, wherein an
enzyme of aequorin (apoaequorin) produced by biosynthesis
in E.coli is used and wherein a substrate employed is
coelenterazine or its analogues.

25 According to another aspect of this invention, a
recombinant DNA molecule is characterised by comprising
biologically pure aequorin synthesised by reacting
coelenterazine or its analogues with apoaequorin.

30 Preferably, in carrying out the method of the
invention, the aequorin enzyme (apoaequorin) is produced
using the aequorin cDNA obtained by the biosynthesis
method disclosed in the above-mentioned patent application
and then, by adding coelenterazine as a substrate to the
enzyme, aequorin having the same properties as natural
aequorin is obtained. The luminescence shown by various
35 metals when reacted with this synthesised aequorin is

1 measured, in order to detect and/or measure the amount of
such metals. As a result, the presence of Ca^{2+} , Sr^{2+} and
 Sm^{3+} can be observed. For Cd^{2+} , its presence can be
confirmed from the inhibitory rate of luminescence of Ca^{2+} .
5 Lanthanoids such as La^{3+} , Tb^{3+} and Yb^{3+} also can be
detected.

The following description serves to illustrate
the invention more specifically.

(1) A method for producing aequorin (apoaequorin) in
10 E.coli.

(1) Insertion of aequorin genes into an expression vector
having a promoter.

As the expression vector into which the aequorin
gene is inserted, the vector in which the promoter is
15 cloned is used. As the expression vector, pUC9 can be
cited as an example. As the promoter, lac, tac or trp
derived from E.coli or PL of the λ phage can be used, by
way of example.

Firstly, pDR540 having the promoter is digested
20 by means of the restriction enzyme BamHI-HindIII. The
resulting fragment containing the promoter is then separated
and extracted by an electrophoresis method, e.g. in 8%
acrylamide gel. By using the promoter thus extracted, the
expression vector (piC9) can be obtained by cloning at the
25 BamHI-HindIII site of the vector.

Then, after digesting this expression vector
with HindIII, its terminals are repaired by E.coli DNA
polymerase (Klenow fragment) in the presence of dATP,
dGTP, dCTP and dTTP, both the terminals are ligated with T_4
30 ligase and an expression vector, piC10, is thus obtained.

In preparing to bind the promoter in the piC10
with the aequorin cDNA gene, a synthetic nucleotide as a
linker is cloned to piC10. Thus, the synthetic nucleotide
linkers AR(5'GATCGATGGTCA-3') and AQ(5'AGCTTGACCATC-3')
35 are first prepared, by a known synthetic method, and then
annealed, after which phosphorylation of their 5'-terminals

-4-

1 is carried out using T_4 nucleotide kinase in the presence
of ATP. The nucleotides thus phosphorylated are sub-
sequently cloned, so as to construct plasmids in the form
of repetition units of the linker at the BamHI-EcoRI site
5 of the piC10, which has been constructed as mentioned
above, and a recombinant DNA piC11 is so obtained.

Next, a HindIII-EcoRI fragment is separated from
aequorin cDNA clone pAQ440 (see Japanese Patent Application
59-176125), this fragment is then inserted into the
10 HindIII-EcoRI site of the above piC11, and the desired
piQ5, namely bacteria containing a promoter in which the
apoaquorin gene has been transformed, is obtained. This
piQ5 has the ability to produce aequorin proteins of
natural type.

15 (2) Insertion of aequorin genes into an expression vector
(pUC9, 9-1, 9-2) having a lac promoter in order to produce
aequorin proteins of a fused type.

In a similar manner to that described in (1)
above, PstI-EcoRI and HindIII-EcoRI fragments are separated
20 from the cDNA clone pAQ440 and purified. Further, ex-
pression vectors pUC9, 9-1, 9-2 having a lac promoter
prepared by a similar method to that described in (1)
above are obtained. Each of these fragments is cloned at
the restriction enzyme site of each of these vectors to
25 produce piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE. All of these
piQ plasmids are under the control of the lac promoter and
thus comprise aequorin proteins of the fused type having
8-amino acid residues at an N-terminus.

(3) Production of proteins having aequorin activity using
30 E.coli.

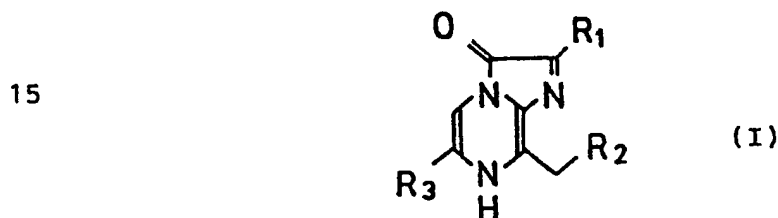
Each plasmid cloned to the expression vectors
obtained by the methods of (1) and (2) is transformed in a
strain, such as E.coli (D 1210), and aequorin proteins are
produced. In this method, for example, E.coli strains
35 containing plasmids are added to a certain volume of LB

1 broth containing a given concentration of ampicillin and
the E.coli strains are cultured. Subsequently, an ex-
pression-inducible reagent is added to the culture medium
and incubation is continued. The resulting culture medium
5 is separated by centrifugation and the resulting cells are
collected and washed with water.

(2) A method for synthesising coelenterazine and its
analogues.

10 In this method, coelenterazine obtained by a
known method can be used.

The coelenterazine used here has the following
general formula:



20 Coelenterazine of the natural type is represented
by the formula wherein R_1 is $p\text{-CH}_2\text{C}_6\text{H}_4\text{OH}$, R_2 is $\text{-C}_6\text{H}_5$ and R_3
is $p\text{-C}_6\text{H}_4\text{OH}$.

Coelenterazine compounds represented by the
formula (I) can be obtained by a known method, for example,
the method disclosed by Inoue et al. (Published by Japan
25 Chemical Society, Chemistry Letters, 141-144, 1975).

Further, as analogues of coelenterazine, analogue
1, represented by the formula (I) wherein R_1 is $\text{-CH}_2\text{C}_6\text{H}_5$,
 R_2 is $\text{-C}_6\text{H}_5$ and R_3 is $p\text{-C}_6\text{H}_4\text{OH}$, or analogue 2, represented
by the formula (I) wherein R_1 is -CH_3 , R_2 is $\text{-C}_6\text{H}_5$ and R_3
30 is $p\text{-C}_6\text{H}_4\text{OH}$, can be used. These analogues can be obtained
by a known method, for example, the method disclosed by
Halt et al. (Biochemistry, 18, (11) 2204-2210, 1979).

(3) A method for detecting metals.

35 Metals are detected by the following method, for
example.

1 The enzyme solution obtained by extraction from
 E.coli using the above process (3) is used. After dissolv-
 ing the enzyme solution in Tris-HCl buffer solution
 containing EDTA, coelenterazine as a substrate and 2-
5 mercaptoethanol are added to the solution and it is then
 allowed to stand on ice, while aequorin is reproduced. A
 sample to be analysed is poured into the solution contain-
 ing aequorin thus reproduced, and the resulting mixed
 solution is transferred to the reaction cell of a spectro-
10 photometer. A sample to be measured is then injected into
 the cell and the quantity of luminescence produced is
 measured.

 By using this invention, metals can be detected
 by means of aequorin produced in E.coli by the recombinant
15 DNA method, using coelenterazine or its analogues as the
 substrate. Therefore, without using natural aequorin,
 which is difficult to obtain, metals can be detected by
 readily available synthetic aequorin produced in E.coli,
 and so can be easily, efficiently and economically detected.

20 The following non-limitative examples illustrate
 this invention more specifically.

EXAMPLE 1

Production of aequorin (apoaequorin) from E.coli

Construction of piC10:

25 The plasmid pDR540 having a tac promoter (pro-
 duced by PL Pharmacia Co. Ltd.) was digested by the
 restriction enzyme BamHI-HindIII and then the resulting
 fragment containing the tac promoter of 92bp was separated
 and extracted by the electrophoresis method using acryl-
30 amide. Then, the BamHI-HindIII site of the resultant
 vector pUC9 was cloned and piC9 was constructed. Then, in
 order to remove the HindIII site of the expression vector
 piC9, firstly the HindIII was digested and then, after
 repairing the terminals of the vector by E.coli DNA
35 polymerase (Klenow fragment) in the presence of dATP,
 dGTP, dCTP and dTTP, both the terminals were ligated with

1 T₄ ligase and piC10 was thus constructed.

EXAMPLE 2

Construction of piC11:

5 In order to bind the aequorin cDNA gene and the
promoter, synthetic linker nucleotides of AR(5'GATCGATGGTCA-3')
and AQ(5'AGCTTGACCATC-3') were prepared. After these
nucleotides were annealed, the 5' terminals were phos-
phorylated with T₄ nucleotide kinase in the presence of
ATP. The nucleotides thus phosphorylated were subsequently
10 cloned, so as to construct in the form of repetition units
of the linker at the BamHI-EcoRI site of the piC10 (EXAMPLE
1), and piC11 was constructed.

EXAMPLE 3

Construction of piQ5:

15 The HindIII-EcoRI fragment was separated and
prepared from aequorin cDNA clone pAQ440, the fragment was
inserted into the HindIII-EcoRI site of the above piC11,
and piQ5 was thus obtained. This piQ5 can reproduce
aequorin protein of the natural type.

20 EXAMPLE 4

Construction of piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE

 After PstI-EcoRI and HindIII-EcoRI fragments
were separated and purified from cDNA clone pAQ440, these
fragments were cloned at the restriction enzyme site of
25 expression vectors pUC9, 9-1, 9-2, having a lac promoter,
and piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE were constructed.

 All of these piQ fragments were under the control of the
lac promoter and so comprised aequorin proteins of the
fused type having 8-amino-acid residues at an N-terminus.

30 EXAMPLE 5

Production of proteins having aequorin activity using
E.coli:

 The above E.coli strains containing plasmids and
having 1/100 volumes, obtained by culturing for 12 hours,
35 were added to 10 ml of LB broth containing 50 µg/ml

1 of ampicillin. The E.coli strains were cultured for 2
hours at 37°C, subsequently an expression-inducible
reagent IPTG (isopropyl-β-thiogaractopylanocide) was
added, to obtain a final concentration of 1 mM, and
5 incubation was continued for 4 hours at 42°C.

The resultant culture medium was separated by
centrifugation at 5000 rpm for 10 minutes (Hitachi RP 20)
and the cells were collected and washed with 5 ml of M9
salt medium. After resolving the washed cells in 2.5 ml
10 of 20 mM Tris-HCl buffer (pH 7.6) containing 10 mM of
EDTA, the cells were destroyed by sonication (60 seconds),
the mixture was centrifuged at 10,000 rpm for 10 minutes
and the resulting supernatant was used as an enzyme
solution for detecting metals.

15 EXAMPLE 6

Synthesis of coelenterazine and its analogues

Coelenterazine and its analogues were synthesised
by the method disclosed by Inoue et al. (Published by
Japan Chemical Society, Chemistry Letters, 141-144, 1975)
20 and by the method disclosed by Halt et al. (Biochemistry,
18, (11) 2204-2210, 1979).

EXAMPLE 7

Method for detecting metals

The enzyme solution obtained by the above method
25 was dissolved in 30 mM of Tris-HCl buffer solution contain-
ing 10 mM of EDTA, 1 µg of the same type of coelenterazine
substrate as the natural type, obtained by the synthesis
method disclosed by Inoue et al., and 5 µl of 2-mercapto-
ethanol were added to the solution to obtain 1 ml of total
30 volume. The solution was allowed to stand for 1 hour in
an ice bath and aequorin was reproduced. Then, the
concentration of metals was measured.

Each sample containing CaCl₂, SrCl₂ and SmCl₃
(1.5 ml, 30 mM) was poured into the solution of aequorin
35 thus reproduced, the solution was transferred to the

-9-

1 reaction cell of a spectrophotometer (Mitchell-Hasting
photometer) and the luminescence of the sample was measured
(1). Further, 30 mM of CaCl_2 was injected into the cell
and the luminescence was measured again (2).

5 The results are shown in the following table:

Metal ion	(1)	(2)
Ca^{2+}	8.3	0
Sr^{2+}	4.9	0
Sm^{3+}	3.6	0
Mn^{2+}	0	8.8
Mg^{2+}	0	8.4
Pb^{2+}	0	8.3
Cd^{2+}	0	0.9

20 In the table, the units of the numerals are $\times 10^{-11}$
photo/sec.

It is apparent from the above table that the metal
ions Ca^{2+} , Sr^{2+} and Sm^{3+} can be detected and the presence
of Cd^{2+} can be also detected because the inhibitory rate
25 of Cd^{2+} is 90%.

Metals were detected using synthesised coelenterazine
of the natural type in the above examples. Further, when
the metals were detected by using analogues of coelenter-
azine, similar results were obtained.

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1 CLAIMS:

5 1. A method for the detection of metals by measuring the luminescence of a metal in the presence of aequorin, characterised in that the aequorin used has been reproduced by adding coelenterazine or an analogue thereof to an enzyme of aequorin (apoaequorin).

10 2. A method as claimed in claim 1, wherein the aequorin enzyme (apoaequorin) is produced in E.coli by a biosynthesis method.

 3. A method as claimed in claim 2, wherein the biosynthesis method is a recombinant DNA technique and the aequorin enzyme (apoaequorin) is produced by using aequorin cDNA.

15 4. A method as claimed in claim 1, 2 or 3, wherein the metal detected is selected from Ca^{2+} , Sr^{2+} and Sm^{3+} , Cd^{2+} and the lanthanoids.

 5. A method as claimed in claim 4, wherein the lanthanoids are selected from La^{3+} , Tb^{3+} and Yb^{3+} .

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EUROPEAN SEARCH REPORT

Application number

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	EP-A-0 137 515 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION) * Pages 9-11 *	1,4	C 12 Q 1/00 C 12 N 15/00
P,X	--- EP-A-0 187 519 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION) * Abstract; page 2, column 1, lines 1-17; page 17, column 2, lines 1-17; pages 29-30 *	2,3	
Y		1,4	
Y	--- TRENDS IN ANALYTICAL CHEMISTRY, vol. 1, no. 16, December 1982, pages 378-383, Elsevier Scientific Publishing Co., Cambridge, GB; F.G. PRENDERGAST: "The use of photoproteins in the detection and quantitation of Ca ²⁺ in biological systems" * Page 378, column 1, lines 30-33; page 378, column 2, lines 1-45; pages 379-380 *	1,4	
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-08-1987	Examiner MEYLAERTS H.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

0245093



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Application number

EP 87 30 4051

Page 2

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A	BIOLOGICAL ABSTRACTS, vol. 80, no. 8, 1985, page 474, abstract no. 68408, Philadelphia, Pasadena, US; O. SHIMOMURA et al.: "Halistaurin, phialidin and modified forms of aequorin as calcium indicator in biological systems", & BIOCHEM J. 228(3), 745-750, 1985 * Abstract *	1	
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P, X	--- BIOCHEMISTRY, vol. 25, no. 26, 30th December 1986, pages 8425-8429, American Chemical Society, Easton, PA, US; S. INOUE et al.: "Expression of apoaquorin complementary DNA in Escherichia coli" * Whole document *	2, 3	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-08-1987	Examiner MEYLAERTS H.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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